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13. ABSTRACT *(Maximum 200 words)*
We described first contact adhesion for cells of Stauroneis decipiens, marine diatoms known to be involved in the formation of primary biofilms, and developed an adhesion assay to test agents that might alter adhesion or fouling. We raised monoclonal antibodies to the adhesive mucilage of S. decipiens. We have detected a suite of extracellular proteoglycans that contain common antigenic epitopes and are located at the site of initial attachment of the diatoms to their substrate and in the trails of sticky mucilage that they secrete. The ability of one antibody (StF.H4) to inhibit adhesion and motility suggests that the epitope bound by this antibody is likely to be close to the adhesive domain(s) of the molecule, and that this antibody should be useful in defining the adhesive site. We are chemically characterizing the proteoglycans, both the carbohydrate and protein, and are close to determining the amino acid sequence for two of the proteins. The atomic force microscope is proving to be a valuable tool in characterizing diatom adhesion trails in the hydrated form, and for quantifying many of its adhesive features on different surfaces.

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FINAL REPORT

Grant #: N00014-94-1-0460 R & T Code 341w006

PRINCIPAL INVESTIGATOR: Rick Wetherbee and Tony Bacic

INSTITUTION: University of Melbourne

GRANT TITLE: Identification and characterisation of the molecule(s) responsible for cell-substrate adhesion in marine raphid diatoms

AWARD PERIOD: 1 February 1994 to 30 September 1997

OBJECTIVE: To investigate the structure and secretion of adhesive molecules from marine raphid diatoms, as well as . other components of the "adhesive complex" of diatoms, which connect the extracellular matrix to the cell membrane and ultimately to the diatom cytoskeleton. To utilize atomic force microscope (AFM) to characterise and quantify the adhesive and elastic properties of diatom adhesive (glycoproteins or proteoglycans) in the hydrated form, and on different substrata.

APPROACH: Monoclonal antibodies were raised to cell surface-associated molecules of the marine raphid diatom *Stauroneis* sp. These antibodies were used as probes to identify the adhesive molecule(s) in the extracellular mucilage secreted by the diatom, and to assist in their isolation. Once isolated, the structure of the protein and carbohydrate portions of the adhesive proteoglycans were characterized using a variety of chemical and enzymatic techniques, and work is continuing to determine the adhesive binding domain, whether protein, polysaccharide, or both. The amino acid sequence data is being used to clone the gene(s) encoding the protein portion(s) of the adhesives. Understanding the structure of these adhesives will enable the design of experiments to determine the molecular basis of their adhesive properties. Structural and physical properties of hydrated diatom adhesives are being investigated with the AFM, and are being linked into an integrated study of the characterisation of adhesive domains in *Stauroneis*.

ACCOMPLISHMENTS: We described the initial contact of *Stauroneis* sp. cells onto microscope slides. Cells typically settle on their sides (girdles), not on their raphes, and are only weakly attached in this position. Within one minute, most cells pull themselves up onto their raphes where they become tightly adhered to the substratum and often become motile. First-contact adhesion occurs at the raphe in this diatom. These observations were used to develop a two-fold adhesion assay based firstly on the ability of cells to manoeuvre onto their raphes, and secondly to glide at the rate of control populations of cells.

We raised monoclonal antibodies to the adhesive mucilage of *Stauroneis decipiens*. Two of these antibodies bind to four proteoglycans (molecular weight 87, 112 and >200 kDa) in the adhesive secretions of the raphe and trails of the diatoms. We have also observed that one of the antibodies inhibits diatom adhesion to the substratum and adhesion mediated cell gliding.

As part of the program directed at characterising the proteoglycans extracted from *Stauroneis decipiens* and the epitopes recognised by our monoclonal antibodies, we are characterising the structure of the carbohydrate portion. The work so far has concentrated on determination of the component neutral sugars and their linkages. Acid hydrolysis of the molecule followed by reduction with $NaBD_4$ and analysis by gas chromatography-mass spectrometry (GC-MS) have revealed 14 neutral sugars, including pentoses (xylose, ribose, and arabinose), hexoses (galactose, glucose, and mannose), 6-deoxyhexoses (rhamnose and fucose), mono-*O*-methylated sugars (3-*O*-methylxylose, 3-*O*-methylfucose, 2-*O*-methylgalactose, 4-*O*-methylgalactose, and 3-*O*-methylmannose), and one aminohexose (glucosamine). The dominant neutral sugar is xylose (45 mol%), followed by galactose (14 mol%) and rhamnose (12.5 mol%). The remaining neutral sugars together account for approximately one-third of the total neutral sugars. Anhydrosugars were not detected by application of the reductive hydrolysis procedure, which assists the recovery and characterisation of acid-labile anhydrosugars.

The linkage patterns of the neutral sugars were analysed by CD_3I-methylation of the triethylamine salt of the proteoglycan. The most salient feature of the neutral sugars is the high proportion of terminal residues (accounting for some 45 mol% of the neutral sugars, approximately half of which occur as terminal xylopyranosyl residues). These terminal residues are of interest to us because they may form the epitopes recognised by the monoclonal antibodies. The proportion of available branch points on the remaining neutral sugars is considerably less (approximately 11 mol%) than the terminal residues, an observation which suggests much of the branching occurs on as yet uncharacterised carbohydrate units, such as uronic acids. The monoclonal antibodies were initially raised to proteoglycans extracted in 8 M urea. We have since been able to significantly increase the yield of the proteoglycans by extraction with 8 M urea in 50% formic acid. We were concerned, however, with the effect of the extraction conditions on the carbohydrate chemistry. For comparison, the proteoglycan was also extracted with bicarbonate to preserve acid-labile anhydrosugars, if present. The neutral sugar composition of the proteoglycan extracted under these three conditions was found to be essentially the same.

We have now completed the primary structure determination of the carbohydrate portion of the proteoglycan with the analysis of the acidic sugars. Following carboxyl reduction and GC-MS analysis, the primary acidic sugar was identified as unesterified glucuronic acid (GlcA; 6-7 mol %) with the possibility of a trace amount of guluronic acid (GulA). The linkage analysis has not been performed. together with the low levels of sulphate (~1% w/w), these results indicate a charged proteoglycan.

The proteoglycans have also been deglycosylated with anhydrous HF and the resulting peptides separated by SDS-PAGE. These deglycosylated peptides were used to demonstrate that the antibodies raised to the adhesive proteoglycans recognize carbohydrate epitopes. These HF-deglycosylated peptides were excised from the gels and following "in-gel" trypsin digestion the resulting peptides were subjected to amino

acid sequencing using either Edman chemistry or LC-MS/MS. Degenerate primers were designed to these sequences and have been used in PCR-based reactions to amplify fragments from a Stauroneis library. These PCR products are being sequenced and used to clone the cDNA corresponding to the peptide sequences. This should lead to the deduction of the sequence of the protein backbone of the adhesive proteoglycans in the near future. This will enable us to make direct comparisons with the other known eukaryotic cell surface adhesion proteins (e.g., integrins) to establish whether there is any conservation of adhesion domains in such divergent organisms.

The AFM is being operated in contact or tapping mode in either air or under physiological conditions (culture medium) to image the sticky trails of *Stauroneis*. Using contact mode on 'old' trails (> 24h after deposition), we have shown for the first time that the trails are persistent, lack obvious sub-structure, and are uniformly 3-5 nm in height. We are currently using tapping mode to characterise the freshly deposited, pliant, and fragile trails in the hydrated state: these trails are extremely sticky. The AFM is also being used to quantify the adhesive and elastic properties of adhesives (glycoproteins/ proteoglycans) by taking force curve measurements, and measuring the electrostatic charge of the trails by assessing attraction/repulsion reactions with a silica sphere attached to the cantilever tip. We have shown that trail material remains very sticky when separated from the cell (and adhesion complex), but cures within a few minutes and is no longer sticky.

CONCLUSIONS: We have detected a suite of extracellular proteoglycans that contain common antigenic epitopes and are located at the site of initial attachment of the diatoms to their substrate and in the trails of sticky mucilage that they secrete. The ability of StF.H4 antibody fragments to inhibit adhesion and motility suggests that the epitope bound by this antibody is likely to be close to the adhesive domain(s) of the molecule, and that this antibody should be useful in defining the adhesive site. The AFM is proving to be a valuable tool in characterising diatom adhesive in the hydrated form and for quantifying many of its adhesive features.

SIGNIFICANCE: We have taken the first steps toward characterizing a diatom adhesive, which is critical to our aim of understanding the mechanism of adhesion and how the processes of adhesion might be overcome. Once the determinants of adhesion are defined, we can compare them to a diverse number of organisms. If generic adhesion motifs exist within the range of bioadhesives, they may be used to develop new adhesives, or strategies to modify and/or prevent adhesion.

AWARD INFORMATION: Phycological Society of America "Provasoli Award" for 1997.

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